Epigallocatechin 3-gallate suppresses interleukin-1 -induced inflammatory responses in intervertebral disc cells in vitro and reduces radiculopathic pain in rats

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EPIGALLOCATECHIN 3-GALLATE SUPPRESSES INTERLEUKIN-1β-INDUCED INFLAMMATORY RESPONSES IN INTERVERTEBRAL DISC CELLS *IN VITRO* AND REDUCES RADICULOPATHIC PAIN IN RATS

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Abstract

Intervertebral disc (IVD) disease, which is characterised by age-related changes in the adult disc, is the most common cause of disc failure and low back pain. The purpose of this study was to analyse the potential of the biologically active polyphenol epigallocatechin 3-gallate (EGCG) for the treatment of painful IVD disease by identifying and explaining its anti-inflammatory and anti-catabolic activity. Human IVD cells were isolated from patients undergoing surgery due to degenerative disc disease (n = 34) and cultured in 2D or 3D. An inflammatory response was activated by IL-1β, EGCG was added, and the expression/activity of inflammatory mediators and pathways was measured by qRT-PCR, western blotting, ELISA, immunofluorescence and transcription factor assay. The small molecule inhibitor SB203580 was used to investigate the involvement of the p38 pathway in the observed effects. The analgesic properties of EGCG were analysed by the von Frey filament test in Sprague-Dawley rats (n = 60). EGCG significantly inhibited the expression of pro-inflammatory mediators and matrix metalloproteinases *in vitro*, as well as radiculopathic pain *in vivo*, most probably by modulation of the activity of IRAK-1 and its downstream effectors p38, JNK and NF-κB.

Keywords: Epigallocatechin 3-gallate, intervertebral disc degeneration, low back pain, IRAK-1, p38, NF-κB, inflammation, radiculopathy, EGCG.

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Introduction

Intervertebral disc (IVD) degeneration is a normal part of the aging process that starts in the late second or early third decade (Dagenais *et al.*, 2008). Although often asymptomatic, specific histological, biochemical and functional changes have been observed during IVD degeneration. These changes are consistent with pain generation and disc degeneration has been suggested as the most common cause of low back pain in adults (Dagenais *et al.*, 2008). Costs associated with health services for spinal problems have been estimated at 85.9 billion USD in 2005 in the United States (Dagenais and Haldeman, 2012; Martin *et al.*, 2008) and many studies have shown that indirect costs related to reduced productivity and subsequent impact on national economy are also significant (Dagenais *et al.*, 2008).

Degeneration of the IVD occurs due to an imbalance between anabolic and catabolic processes, leading to a loss in collagen and proteoglycan and a concomitant reduction in water content by enhanced exposure to matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) (Bibby *et al.*, 2005; Le Maitre *et al.*, 2007a; Pockert *et al.*, 2009; Roberts *et al.*, 2006; Vo *et al.*, 2013). These degenerative processes lead to mechanical dysfunction and altered stress distribution in the tissue, hence increasing the risk for load-induced structural failure in the annulus fibrosus (AF), so-called clefts, tears or fissures. Under these circumstances, pain sensation can be provoked by leakage of nucleus pulopsus (NP) material through the AF (*i.e.* disc herniation) and consequent irritation of spinal nerves (radiculopathy) and/or nerve infiltration into the compromised disc (nociception).

However, even without a disc prolapse, the degenerated IVD can be painful, especially if high levels of pro-inflammatory mediators are secreted. Cytokines, *e.g.* from the interleukin-1 (IL-1) superfamily, not only irritate nerve endings in the AF (Richardson *et al.*, 2007), but also stimulate the production of matrix-degrading enzymes, hence further worsening the degenerative processes (Le Maitre *et al.*, 2005; Shamji *et al.*, 2010; Wuertz *et al.*, 2012). The healthy IVD is excluded from the development of immunologic tolerance as an immune-privileged organ with no access to systemic circulation (Sun *et al.*, 2013). In contrast, diseased human discs are heavily invaded by blood vessels and nociceptive nerve fibres (Brisby, 2006), so that anti-angiogenic and anti-neurogenic factors

are of therapeutic interest in terms of nociceptive pain (Sun *et al.*, 2013). Once NP extrudes from the IVD to the systemic circulation, the immune system recognises it as a foreign body, leading to autoimmune reactions conducted by activated B and cytotoxic T lymphocytes, which may further enhance the destruction of NP tissue inside the disc (Geiss *et al.*, 2007). On the other hand, some immune cells (macrophages, mast cells) might be beneficial to the regrowth of the IVD wounds and immune privilege maintenance at early stage of the degeneration (Peng *et al.*, 2006) as well as in the absorption of herniated NP (Doita *et al.*, 2001; Gronblad *et al.*, 1994; Sun *et al.*, 2013). When conservative treatment (physical therapy, pharmacological treatment) fails, patients with disc herniation or painful degenerative disc disease are likely to undergo surgical interventions; discectomy in case of herniation, spinal fusion or disc replacement in case of disc disease. Imaging techniques such as MRI are a valuable tool to identify the source of pain in disc herniation patients, however in patients with degenerative disc disease, identification of the painful disc is error-prone and removal of the degenerated tissue has a negative impact on disc height or load-bearing capacity (Kandel *et al.*, 2008). In the case of spinal fusion, a consequent risk for adjacent segment degeneration, with the need of additional surgeries, is also significant (Disch *et al.*, 2008; Kandel *et al.*, 2008; Richardson *et al.*, 2007).

Therefore, currently used procedures for the treatment of degenerative disc disease are not optimal. New, less invasive but more targeted strategies are being developed (Richardson *et al.*, 2007). NP replacement, *e.g.* by the injection of biocompatible hydrogels with or without cells may have the potential to restore normal disc height and load distribution, as well as limit degenerative changes in adjacent discs (Richardson *et al.*, 2007). Recently, tissue-engineered IVDs that formed a functional motion segment were used to replace the degenerated discs in rodent caudal spine (Bowles *et al.*, 2011) and chemical crosslinking was applied to stabilise the AF tissue *in vitro* (Hedman *et al.*, 2006). Anti-inflammatory and anti-catabolic substances that target the metabolism and inflammatory signalling within the IVD also represent a new, interesting treatment option (Klawitter *et al.*, 2012a; Klawitter *et al.*, 2012b; Sinclair *et al.*, 2011; Wuertz *et al.*, 2011).

Epigallocatechin 3-gallate (EGCG) is a biologically active polyphenolic catechin present in green tea. EGCG forms 40-60 % of all green tea catechins and is reported to be responsible for the major health benefits of green tea due to its anti-oxidant, anti-aging and anti-inflammatory properties, which are exhibited by direct or indirect interaction with many molecules and signalling transduction pathways in cells (Bode and Dong, 2009; Singh *et al.*, 2011). EGCG was shown to have beneficial effects for a number of clinical conditions, including cancer, obesity, atherosclerosis, diabetes, liver and neurodegenerative diseases (Aggarwal and Shishodia, 2006; Smid *et al.*, 2012; Suzuki *et al.*, 2012), with cell-type specific effects and modes of action (Singh *et al.*, 2011; Suzuki *et al.*, 2012). Although EGCG has chemopreventive and anticancer effects and acts synergistically with several anticancer drugs (Khan and Mukhtar, 2007), it has also been shown that EGCG can block the function of boronic acid proteasome inhibitors (Golden *et al.*, 2009) and reduce the bioavailability of the novel oral multi-target tyrosine kinase inhibitor sunitinib (Ge *et al.*, 2011). Moreover, the consumption of EGCG by pregnant women could increase the risk of innate acute myeloid leukaemia in children, because EGCG inhibits topoisomerase II activity not only in cancer cells, but also in the foetus. This can lead to the translocation at chromosome 11q23 involving the mixed-lineage leukaemia (MLL) gene (Lambert *et al.*, 2007; Strick *et al.*, 2000). Therefore, EGCG is possibly contraindicated in particular cases.

Beneficial effects of EGCG and green tea extract were described in osteoarthritic (OA) chondrocytes (Shen *et al.*, 2012), which bear similarities to IVD cells. It has recently been demonstrated that EGCG inhibits the general IL-1β-induced response, but does not have significant anabolic effects in chondrocytes. The anti-inflammatory effect of EGCG in human chondrocytes is mainly mediated by inhibition of c-Jun N-terminal protein kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity (Ahmed *et al.*, 2004; Akhtar and Haqqi, 2011). EGCG was shown to inhibit NF-κB activation by suppressing the degradation of its inhibitory protein in the cytoplasm (Rasheed *et al.*, 2009). EGCG also reduces the activity of activator protein 1 (AP-1) transcription factor (Ahmed *et al.*, 2004), p38 mitogen-activated protein kinase (MAPK) (Rasheed *et al.*, 2009) and blocks interleukin-1 receptor-associated kinase 1 (IRAK-1) degradation (Akhtar and Haqqi, 2011) in OA chondrocytes. EGCG is able to inhibit the expression of cyclooxygenase 2 (COX-2) and prostaglandin E synthase (PGES) (Ahmed *et al.*, 2002), which are both involved in the development and transduction of inflammation and pain. EGCG also potently blocks the toll-like receptor 4 (TLR4) signalling pathway, which may play an important role in the occurrence and development of neuropathic pain in rats (Kuang *et al.*, 2012). Based on the promising findings of EGCG effects in cartilage, we hypothesise that EGCG may also possess anti-inflammatory and anti-catabolic effects in the IVD and thus have a potential for the treatment of pain and inflammation in degenerative disc disease.

Materials and Methods

Human IVD cell culture preparation

The study was approved by ethic committees Kantonale Ethikkommission Zürich EK-16/2005 and Ethikkommission des Kantons Luzern 1007IVD. Human NP tissue was removed from patients undergoing spinal surgery for degenerative disc disease or disc herniation after informed consent was granted. The details about the donors used in this study are listed in Table 1. Tissue was enzymatically digested using a mixture of 0.2 % collagenase NB4 (17454, Serva, Heidelberg, Germany) and 0.3 % dispase II (04942078001, Roche, Basel, Switzerland) for 4-8 h at 37 °C and isolated primary cells were seeded in Dulbecco's Modified Eagle's Medium (DMEM/F12, D8437, Sigma, St. Louis, MO, USA) supplemented with 10 % foetal calf serum (FCS, F7524, Sigma), penicillin

Table 1. Demographic data of all donors used for the experiments. STEN, discogenic stenosis; DH, disc herniation; DDD, degenerative disc disease; SP, spondylolisthesis; E, ELISA; G, gene expression; W, western blotting; V, viability; TF, transcription factor assay; IC, immunocytochemistry; uk, unknown.

Donor	Sex	Age	Level	Pathology	Grade	Experiment
1	F	43	L4/5	DH	IV	E(3D), G(3D), W
2	F	62	L4/5	SP	V	E(3D), G(3D), W
3	F	49	L5/S1	protrusion	IV	E(3D), G(3D)
4	M	42	C6/7	DH	III	E(3D), G(3D)
5	M	47	L2/3	DH	IV	E(3D), G(3D)
6	F	67	L4/5	DH	IV	E(3D), G(3D)
7	uk	uk	uk	uk	uk	E(2D,3D), G(2D,3D)
8	M	50	L5/S1	SP	III	E(3D), G(3D)
9	uk	uk	uk	uk	uk	E(3D), G(3D)
10	M	41	L4/5	DH	IV	E(2D), G(2D), G(SB), W
11	M	52	L1/2	DH	V	E(2D), G(2D), TF
12	F	53	C6/7	DH	IV	E(2D), G(2D), IS
13	F	46	L5/S1	DH	V	E(2D), G(2D), IS
14	M	42	L4/5	DH	IV	G(2D),W
15	M	39	L4/5	DH	IV	G(2D,3D), G(SB)
16	F	54	L3/4	SP	V	G(2D), TF
17	F	38	L5/S1	DH	IV	G(2D), G(SB), TF
18	M	59	L4/5	DH	IV	G(3D)
19	uk	uk	uk	uk	uk	G(3D), W
20	M	59	L3/4	DH	V	G(2D), W
21	M	57	L5/S1	DH	V	G(2D), W
22	uk	uk	uk	uk	uk	G(2D), G(SB)
23	M	48	L5/S1	DH	IV	G(2D), G(SB), W
24	uk	uk	uk	uk	uk	G(3D), W
25	M	54	L4/5	DH	III	G(2D),W
26	F	49	C4/5	DH	II	V, G(2D)
27	M	62	C5/6-C6/7	STEN	III	V, G(2D)
28	uk	uk	uk	uk	uk	V, G(2D)
29	uk	uk	uk	uk	uk	V, G(2D)
30	F	57	L3/4	SP	III	V, G(2D)
31	M	56	L5/S1	DH	III	V, G(2D)
32	M	63	L2/3	DH	IV	V, G(2D)
33	uk	uk	uk	uk	uk	V, G(2D)
34	F	25	L5/S1	DDD	III	G(2D)

(50 units/mL), streptomycin (50 µg/mL) and ampicillin (125 ng/mL, 15240-062, Gibco, Carlsbad, CA USA) and sub-cultured up to passage 3 using 1.5 % trypsin (15090-046, Gibco). Cells in passage 1-3 that were cultured either adherently (2D) or in alginate beads (3D) were used for experiments.

Alginate beads preparation

Alginate was prepared as a solution of 1.2 % alginic acid sodium salt (180947, Sigma-Aldrich, St. Louis, MO, USA) in 0.9 % sodium chloride, stirred overnight and sterile filtered. Human IVD cells in passage 1-3 were harvested with trypsin and gently mixed with alginate (4 x 10^6 cells per 1 mL of alginate). Alginate-cell suspension was slowly pushed through a sterile syringe with a 21G needle and dropped into a 102 mM calcium chloride solution with constant speed. Beads were formed after 5 min of stirring in the calcium chloride solution and then washed with 0.9 % NaCl and phosphate-buffered saline (PBS). Beads were spread into 6-well plates with cell culture medium containing 50 µg/mL vitamin C (A4403, Sigma) and cultured for 7 d with one medium exchange.

Viability measurement

Non-toxic concentrations of EGCG (E4243, Sigma) were defined using the MTT assay in 2D cell culture. Cells were

seeded in 12-well plates (1 x 10^5 cells/well) and treated with different concentrations of EGCG (0.1-50 μM) in medium with 10 % FCS or without FCS. After 24 and 48 h, fresh MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, M5655, Sigma) solution in PBS (0.5 mg/mL) was added and kept for 4 h in 37 °C. MTT was discarded, cells were lysed in DMSO (D8418, Sigma) and absorbance was measured at 565 nm relative to untreated controls. The non-toxic concentration of 10 μM was chosen for subsequent experiments.

Gene expression analysis (qRT-PCR)

The effect of EGCG on the expression of inflammatory mediators and matrix degrading enzymes was studied in 2D and 3D cell culture models that were pre-treated with IL-1β (211-11, Peprotech, Hamburg, Germany) in order to induce the expression of pro-inflammatory and catabolic genes and hence simulate the changes seen during inflammation-related disc degeneration (Le Maitre *et al.*, 2005; Mengshol *et al.*, 2000). IVD cells were seeded in 6-well plates (3 x 10^5 cells/well) (2D) or alginate beads (3D), serum-starved for 2 h and then exposed to 10 ng/mL IL-1β for 2 h before treatment with 10 μM EGCG. The involvement of the p38 pathway was studied using the small molecule inhibitor of p38 MAPK SB203580 (SB, S8307, Sigma), which was added 2 h after IL-1β pre-stimulation in the concentration of 10 μM to the cells seeded in 2D ($n = 3$ for MMPs and 4 for iNOS and COX-2). After 18 h, RNA was extracted with the Trizol/chloroform method according to the manufacturer instructions (15596-018, Invitrogen, Carlsbad, CA, USA) and 1 μg was reverse transcribed to cDNA using a reverse transcription kit (4374966, Applied Biosystems, Foster City, CA, USA). cDNA was then mixed with primers and master mix (4352042, Applied Biosystems) and gene expression was measured using real-time PCR. Data was analysed by the comparative Cq method ($2^{\Delta\Delta Cq}$ method, housekeeping gene TATA box binding protein, TBP). Primer details are given in Table 2. Results are presented as gene expression relative to IL-1β pre-stimulation (100 %).

Western blotting

IVD cells seeded in 12-well plates (1 x 10^5 cells/well) were serum starved for 2 h and then exposed to 10 ng/mL IL-1β for 2 h before treatment with 10 μM EGCG or 10 μM SB, 15 min for p-p38 ($n = 9$), p-JNK ($n = 3$) and IRAK-1 ($n = 5$), 6 h for COX-2 ($n = 1$). For total protein analysis, cells were lysed in 0.1 % sodium dodecyl sulphate (SDS) buffer, mixed with Laemmli buffer (S3401, Sigma), heated (99 °C, 5 min) and lysates were loaded onto 10 % SDS-polyacrylamide gels. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (RPN303F, GE Healthcare, Little Chalfont, UK) and membranes were blocked in 5 % non-fat milk in Tris-buffered saline-Tween (TBS-T) for 1 h at room temperature. Primary antibodies were applied overnight at 4 °C. After washing in 1 % non-fat milk in TBS-T (3 × 10 min), membranes were incubated with secondary antibody conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. Visualisation was performed on medical X-ray film (28906836, GE Healthcare) using a chemiluminescence kit West Dura (34076, Thermo Scientific, Waltham, MA, USA), films were scanned and scans were processed by GIMP2. Tubulin was used as a loading control. Antibodies and dilutions used: p38 MAPK, 1:1000 (9212, Cell Signaling, Danvers, MA, USA); phospho-SAPK/JNK, 1:1000 (9251, Cell Signaling); phospho-p38MAPK, 1:1000 (9211, Cell Signaling); α-Tubulin, 1:1000 (2144, Cell Signaling); IRAK-1, 1:1000 (4359, Cell Signaling); COX-2, 1:1000 (4842, Cell Signaling) and mouse anti-rabbit IgG HRP, 1:5000 (211-032-171, Jackson Immuno Research, West Grove, PA, USA).

Immunocytochemistry

IVD cells ($n = 2$) seeded onto cover slips in Petri dishes were serum-starved for 2 h and then exposed to 10 ng/mL IL-1β alone or in combination with 10 μM EGCG. After 1 h of simultaneous treatment, cells were washed with PBS (3 × 5 min), fixed with ice-cold methanol (10 min on ice), washed with PBS (3 × 5 min), and blocked with 2 % BSA (A4503, Sigma) with the addition of 5 % goat serum (G9023, Sigma) (1 h at room temperature). Primary antibody p65 (RelA, the large subunit of NF-κB, Santa Cruz, sc-372, 1:200) was applied overnight at 4 °C. The next day, cells were washed with PBS (3 × 5 min) and secondary antibody (CY2 goat anti-rabbit IgG, Jackson Immuno Research, 111-165-144, 1:400) was applied for 1 h at room temperature. After washing in PBS (3 × 5 min), DAPI (4',6-diamidino-2-phenylindole, D9542, Sigma) was applied (5 min), cells were washed and embedded in Mowiol 4-88 (81381, Sigma). Analysis of p65 translocation was performed with a fluorescent microscope (Olympus IX51) and micrographs were processed in ImageJ. Cells without primary antibody were used as a non-specific

Table 2. Primers used for real-time RT-PCR (TaqMan Gene Expression Assays, Applied Biosystems).

Gene	Primer Sequence Number	Base Pairs
TATA box binding protein (TBP)	Hs00427620_m1	91
Interleukin-6 (IL-6)	Hs00174131_m1	95
Interleukin-8 (IL-8)	Hs00174103_m1	101
Matrix metalloproteinase-1 (MMP1)	Hs00233958_m1	133
Matrix metalloproteinase-3 (MMP3)	Hs00968308_m1	98
Matrixmetalloproteinase-13 (MMP13)	Hs00233992_m1	91
Toll-like receptor 2 (TLR2)	Hs00152932_m1	80
Cyclooxygenase-2 (COX-2)	Hs00153133_m1	75
Inducible nitric oxide synthase (iNOS)	Hs01075521_m1	82
Nerve growth factor (NGF)	Hs00171458_m1	102

binding control, untreated cells were used as a negative control, and cells treated with 10 ng/mL IL-1β were used as a positive control for p65 translocation. Antibodies and dilutions used: NF-κB p65,1:200 (sc-372, Santa Cruz, Dallas, TX, USA) and CY2 goat anti-rabbit IgG, 1:400 (111-165-144, Jackson Immuno Research).

Nuclear extraction and transcription factor assay

Nuclear extracts were prepared using a Nuclear Extraction kit (10009277, Cayman, Ann Arbor, MI, USA). Cells (n = 3) were seeded in T75 flasks in the density 5 x 10^6 cells/flask and pre-cultured 24 h. Cells were serum-starved for 2 h and then exposed to 10 ng/mL IL-1β alone or in combination with 10 μM EGCG. After 1 h of simultaneous treatment, nuclear extraction was performed according to the producer's protocol. Briefly, cells were collected into 15 mL pre-chilled tubes, centrifuged 2 times with kit PBS/phosphatase inhibitor solution, and then re-suspended in ice-cold kit hypotonic buffer. After 15 min of incubation, 10 % Nonidet P-40 was added, cells were centrifuged for 1 min at 140 x *g*, and supernatants containing the cytoplasmic fraction were collected. Pellets were further re-suspended in kit complete nuclear extraction buffer by vortexing (15 s), shaking (15 min on ice) and vortexing (15 s). Supernatants containing the nuclear fraction were collected into new pre-chilled tubes after 10 min of centrifugation (16000 x *g*, 4 °C). Protein concentration was determined using a BCA assay kit (23227, Thermo Scientific). NF-κB (p65) Transcription Factor Assay Kit (10007889, Cayman) was used for the assessment of NF-κB (p65) DNA-binding activity in nuclear extracts according to the producer's protocol on a 96-well plate. An equal amount of protein (15 μg/well) was loaded in duplicates for each sample. Provided non-specific binding control, competitor dsDNA control and positive control were used to monitor appropriate assay function. The 96-well plate was incubated 2 h at room temperature, washed with kit wash buffer, and primary antibody was added. After 1 h, the plate was washed, incubated with secondary antibody, washed again and incubated with kit developing solution for 30 min. Finally, kit stop solution was applied and absorbance at 450 nm was measured immediately. The result is presented as NF-κB (p65) DNA-binding activity relative to IL-1β pre-stimulation (100 %).

Enzyme-linked immunosorbent assay (ELISA)

To detect secreted proteins, IVD cells in passage 1-3 were seeded on 6-well plates (3 x 10^5 cells/well) (n = 5) or in alginate beads (n = 9), serum starved for 2 h and then exposed to 10 ng/mL IL-1β for 2 h before treatment with 10 μM EGCG. Cell culture medium was collected after 18 h and the level of IL-6 protein expression was analysed by ELISA according to the producer's protocol (Human IL-6 ELISA set, 555220 with Reagent set B, 550534, BD Biosciences, San Jose, CA, USA). Briefly, 96-well plates were coated by kit capture antibody at 4 °C overnight, washed and blocked in kit assay diluent. After washing, samples with controls and standards were incubated on the plates for 2 h at room temperature. Then plates were washed, kit detection antibody, streptavidin-horseradish peroxidase (HRP) and substrate solution were applied according to the producer's protocol. Absorbance was measured within 30 min at 450 nm with 570 nm correction after the addition of a kit stop solution. Results are presented as protein expression relative to IL-1β pre-stimulation (100 %).

***In vivo* study on pain behaviour**

All animal experiments were carried out under the control of the Animal Care and Use Committee in accordance with local guidelines for animal experiments and government law concerning the protection and control of animals. Female Sprague-Dawley rats (n = 60, 200-250 g) (Japan SLC, Shizuoka, Japan) were used in this study. A combination of 0.3 mL medetomidine hydrochloride (1.0 mg/mL), 0.8 mL midazolam (5.0 mg/mL), and 1.0 mL butophanol tartrate (5.0 mg/mL) was prepared as an anaesthetic. Animals were anaesthetised by intraperitoneal injection of 0.1 mL/100 g body weight of the mixed anaesthetic. Animals were placed in a prone position and the surgical intervention was performed with a stereo operating microscope and microsurgical instruments. An incision was made to the spinal midline, then fascia and multifidus muscle were resected, and the left L5 nerve root and dorsal root ganglion (DRG) were exposed to L5-L6 facetectomy on the left side, with great care taken to avoid trauma to the tissue. In the NP application group (NP group, n = 48), autologous NP was harvested from the tail and applied to the DRG (Kobayashi *et al.*, 2011; Otoshi *et al.*, 2010; Sekiguchi *et al.*, 2011). In the sham-operated group (Sham group, n = 12), the left L5 nerve root and DRG were exposed to L5-L6 facetectomy, but no other procedures were performed. Animals from the NP application group were divided into four groups (n = 12 in each): NP + EGCG 10 μM (0.1 mL of 10 μM EGCG), NP + EGCG 100 μM (0.1 mL of 100 μM EGCG), NP + water (0.1 mL of water as a vehicle), and NP (non-treatment) group. Animals in the treatment groups were injected with 0.1 mL of the designed treatment solution into the underlayer of epineurium, just distal to the NP, before closing the incisions (Kobayashi *et al.*, 2011; Wuertz *et al.*, 2011). Sensitivity to non-noxious mechanical stimuli was tested using the von Frey Filament test. Baseline testing was performed before the start of the experiment to accommodate animals with normal responses. Hind paw withdrawal response to von Frey hair (North Coast Medical, Morgan Hill, CA, USA) stimulation of the plantar surface of the footpads was determined at 2, 7, 14, 21 and 28 d after surgery. Individual rats were placed in an acrylic cage with a mesh floor and allowed to acclimate for 15 min or until cage exploration and major grooming activities ceased. The lateral plantar surface of the operated hind paw, innervated by the L5 nerve, was stimulated with nine von Frey filaments (1.02, 1.4, 2.0, 4.1, 6.1, 8.0, 10.6, 15.4 and 26.0 g) threaded under the mesh floor. The gram ratings for von Frey hairs were based on the ratings supplied by the manufacturer. The filaments were sequentially applied to the paw surface just until the filament bent and was held for 3 s. The response was considered positive if the rat lifted the foot in combination with either licking or shaking of the foot as an escape response (Kobayashi *et al.*, 2011; Wuertz *et al.*, 2011).

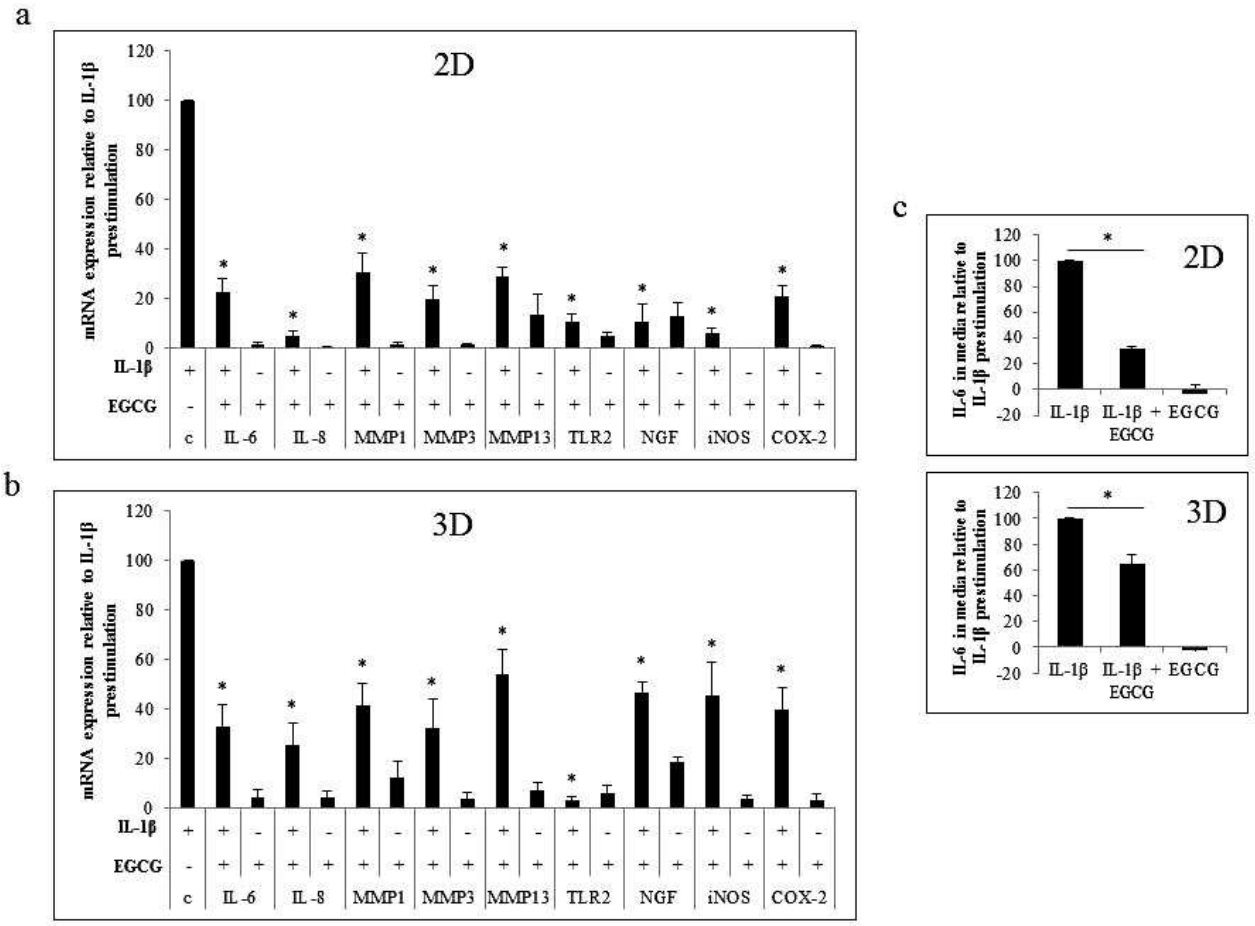

Fig. 1. EGCG exhibits anti-inflammatory and anti-catabolic effects in 2D and 3D IVD cell culture. Cells were pre-stimulated with IL-1β for 2 h before treatment with EGCG and compared to cells treated with IL-1β or EGCG separately. After 18 h, EGCG significantly reduced mRNA expression of IL-6, IL-8, MMP1, MMP3, MMP13, TLR2, NGF, iNOS and COX-2, both in 2D adherent cell culture (**a**) and in 3D alginate beads (**b**). IL-6 protein secretion in cell culture media was also significantly decreased (**c**). Data are presented as mRNA or protein expression relative to IL-1β pre-stimulation. Asterisks indicate statistical significance ($p < 0.05$, Student's *t*-test).

Statistical analysis

Statistical significance between IL-1β and IL-1β+EGCG groups (Figs. 1, 2) as well as between IL-1β and IL-1β+SB groups (Fig. 3) was analysed using Student's *t*-test. Asterisks represent a significance level of $p < 0.05$. For the animal study, behavioural data between the groups over the experimental period (28 d) was statistically evaluated by ANOVA with Bonferroni *post-hoc* testing to correct for multiple comparisons. Asterisks represent a significance level of $p < 0.01$.

Results

EGCG causes a significant decrease in the expression of inflammatory mediators and matrix metalloproteinases in IVD cells cultured *in vitro*

The effect of EGCG on the expression of inflammatory mediators and matrix metalloproteinases was determined in adherent 2D cell culture and in 3D alginate beads on the mRNA level after 18 h. A significant inhibition of mRNA expression of interleukins (IL-6, IL-8), matrix metalloproteinases (MMP1, MMP3, MMP13), toll-like receptor 2 (TLR2), nerve growth factor (NGF), inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) was detected upon EGCG treatment in both, 2D and 3D (Fig. 1a, 1b). The exact values for each gene, including *p*-values, are listed in Table 3. To confirm these observations on the protein level, IL-6 was chosen to be measured in cell culture media by ELISA, due to its strong regulation on the mRNA level and pathological relevance. EGCG treatment significantly decreased IL-6 secretion into the medium, compared to the IL-1β pre-stimulated cells both in 2D (31.26 %, $p = 0.0007$) and 3D (64.45 %, $p = 0.0012$) (Fig. 1c). Displaying the data relative to the IL-1β pre-stimulation (set as 100 %) minimises the inter-donor variation and shows clearly the effect of the studied compound on the acute inflammatory response.

EGCG inhibits IL-1β-induced IRAK-1 degradation and partially blocks NF-κB, p38 and JNK activity

IRAK-1 was reported to be important for the transduction of inflammatory signals (IL-1β, LPS), which cause its phosphorylation and degradation, hence enabling the activation of stress-related signalling molecules NF-κB, p38 and JNK (Janssens and Beyaert, 2003). IL-1β pre-

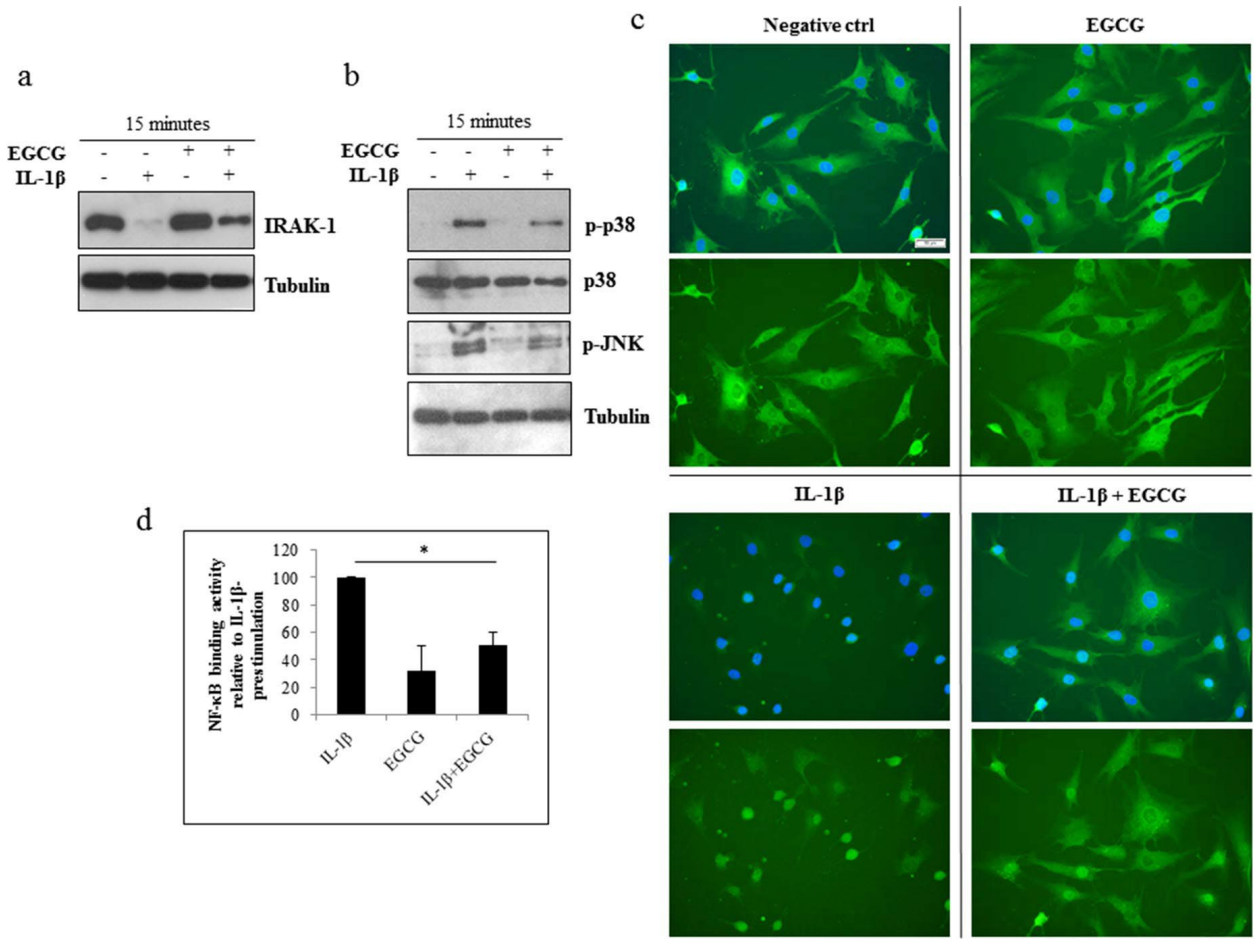

Fig. 2. EGCG inhibits IRAK-1 and NF-κB/p38 /JNK signalling in IVD cells. Cells were pre-stimulated with IL-1β for 2 h before EGCG was added. After 15 min, cells were lysed and western blotting was performed. IRAK-1 degradation (**a**) and p38 and JNK phosphorylation (**b**) were inhibited upon EGCG treatment of IL-1β-stimulated cells. Combination of IL-1β with EGCG partially blocks NF-κB (p65) translocation to the nucleus (**c**) and NF-κB (p65) DNA-binding activity (**d**) after 1 h. Asterisk indicates statistical significance ($p < 0.05$, Student's *t*-test).

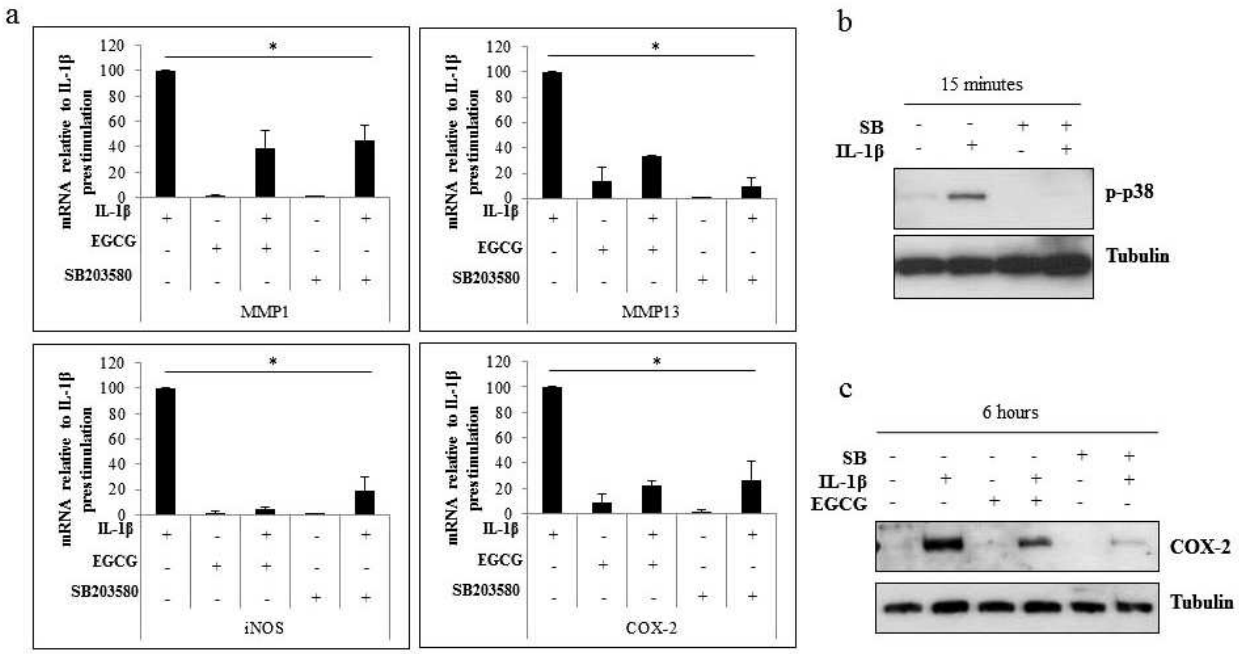

Fig. 3. EGCG supresses p38 MAPK-dependent MMP1, MMP13, iNOS and COX-2 expression in IL-1β pre-stimulated IVD cells. Cells were pre-stimulated with IL-1β for 2 h before treatments with EGCG or SB203580 (SB). Control cells were either untreated or treated with IL-1β, EGCG or SB separately. After 18 h, EGCG and SB203580 both reduced mRNA expression of MMP1, MMP13, iNOS and COX-2 (**a**). Data are presented as mRNA expression relative to IL-1β pre-stimulation. Asterisks indicate statistical significance ($p < 0.05$, Student's *t*-test). The function of p38 MAPK inhibitor SB203580 was confirmed by western blotting (**b**). The addition of EGCG or SB to IL-1β-stimulated cells inhibits p38-dependent COX-2 protein expression (**c**).

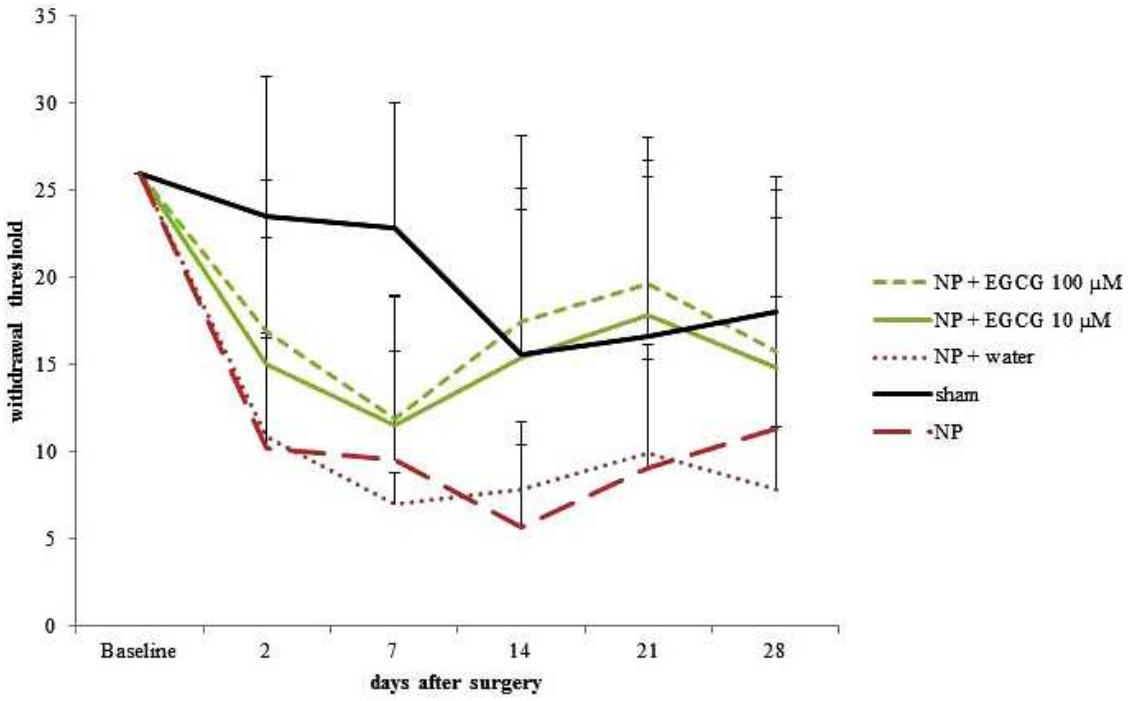

Fig. 4. EGCG inhibits pain behaviour *in vivo*. Pain sensitivity was measured by the von Frey filament test for 28 d. When NP tissue was placed on the DRG without therapeutic treatment (NP group and NP + water group), the mechanical withdrawal thresholds were significantly decreased. EGCG treatment (NP + EGCG 10 μM, NP + EGCG 100 μM) prevented the decrease in the mechanical withdrawal threshold observed in the NP group and NP + water group and restored the threshold level to that observed in the sham group.

stimulated IVD cells were treated with EGCG and the level of IRAK-1, together with the activity of its downstream effectors p38 and JNK, were determined by Western blotting. IRAK-1 was degraded in the cells stimulated with IL-1β, but the level of IRAK-1 degradation was reduced in cells treated with IL-1β + EGCG (Fig. 2a). Phosphorylation of p38 and JNK was also decreased in IL-1β + EGCG-treated cells, compared to IL-1β-stimulated cells, *i.e.* EGCG reduced p38 and JNK activity (Fig. 2b). The effect of EGCG on the activity of the transcription factor NF-κB was studied by immunofluorescence, visualising the translocation of NF-κB subunit p65 (RelA) to the nucleus and by NF-κB (p65) Transcription Factor Assay. The effect of EGCG + IL-1β studied by immunocytochemistry prevented p65 translocation in approximately 50 % of cells. Cells without primary antibody, untreated cells and cells treated with IL-1β were used as controls. As nuclear NF- κB was still detectable in the EGCG + IL-1β-treated cells (Fig. 2c), a Transcription Factor Assay was performed to quantify the nuclear localisation of NF-κB in EGCG + IL-1β-treated cells through measurement of DNA binding activity. Results of the Transcription Factor Assay showed 50.83 % (p = 0.0327) decrease in NF-κB DNA-binding activity upon EGCG treatment (Fig. 2d). Results are presented as NF-κB DNA binding activity relative to IL-1β-stimulated cells (100 %). A suggested mechanism of EGCG action in IVD cells is shown in Fig. 5.

EGCG inhibits p38 MAPK-dependent expression of COX-2, iNOS, MMP1 and MMP13

As shown in Fig. 2, EGCG modulates the IRAK-1 and p38 signalling pathway in IVD cells. In order to reveal which inflammation-related molecules are regulated by p38 MAPK on the gene expression level, a small molecule inhibitor of p38 MAPK, SB203580 (SB), was used. IL-1β pre-stimulated cells were treated with either EGCG or SB and mRNA expression was measured after 18 h. Both, EGCG and SB treatment significantly inhibits COX-2 (SB: 26.79 %, p = 0.0151), iNOS (SB: 19.39 %, p = 0.0042), MMP1 (SB: 44.54 %, p = 0.0494) and MMP13 (SB: 6.66 %, p = 0.0042) expression compared to IL-1β pre-stimulated cells (100 %) ($p < 0.05$) (Fig. 3a). The ability of SB to inhibit p38 activity was confirmed by Western blotting (Fig. 3b). The regulation of COX-2 expression by EGCG and SB was proved also at the protein level by Western blotting (Fig. 3c). The expression of other studied genes (IL-6, IL-8, MMP3, TLR2, NGF) is only partially p38 dependent or p38 independent (data not shown).

Table 3. Percentage of mRNA expression in samples treated with IL-1β + EGCG, relative to IL-1β pre-stimulation, shown in Fig. 1.

	2D		3D	
Gene	**Expression, *p*-Value**		**Expression, *p*-Value**	
IL-6	22.72 %	$p < 0.0001$	32.79 %	$p = 0.0002$
IL-8	4.99 %	$p < 0.0001$	25.69 %	$p = 0.0002$
MMP1	30.40 %	$p < 0.0001$	41.40 %	$p = 0.0013$
MMP3	19.73 %	$p < 0.0001$	32.55 %	$p = 0.0020$
MMP13	28.93 %	$p < 0.0001$	54.08 %	$p = 0.0096$
TLR2	10.43 %	$p < 0.0001$	3.25 %	$p = 0.0001$
NGF	10.67 %	$p = 0.0002$	46.79 %	$p = 0.0003$
iNOS	5.91 %	$p < 0.0001$	45.44 %	$p = 0.0096$
COX-2	20.68 %	$p < 0.0001$	39.62 %	$p = 0.0067$

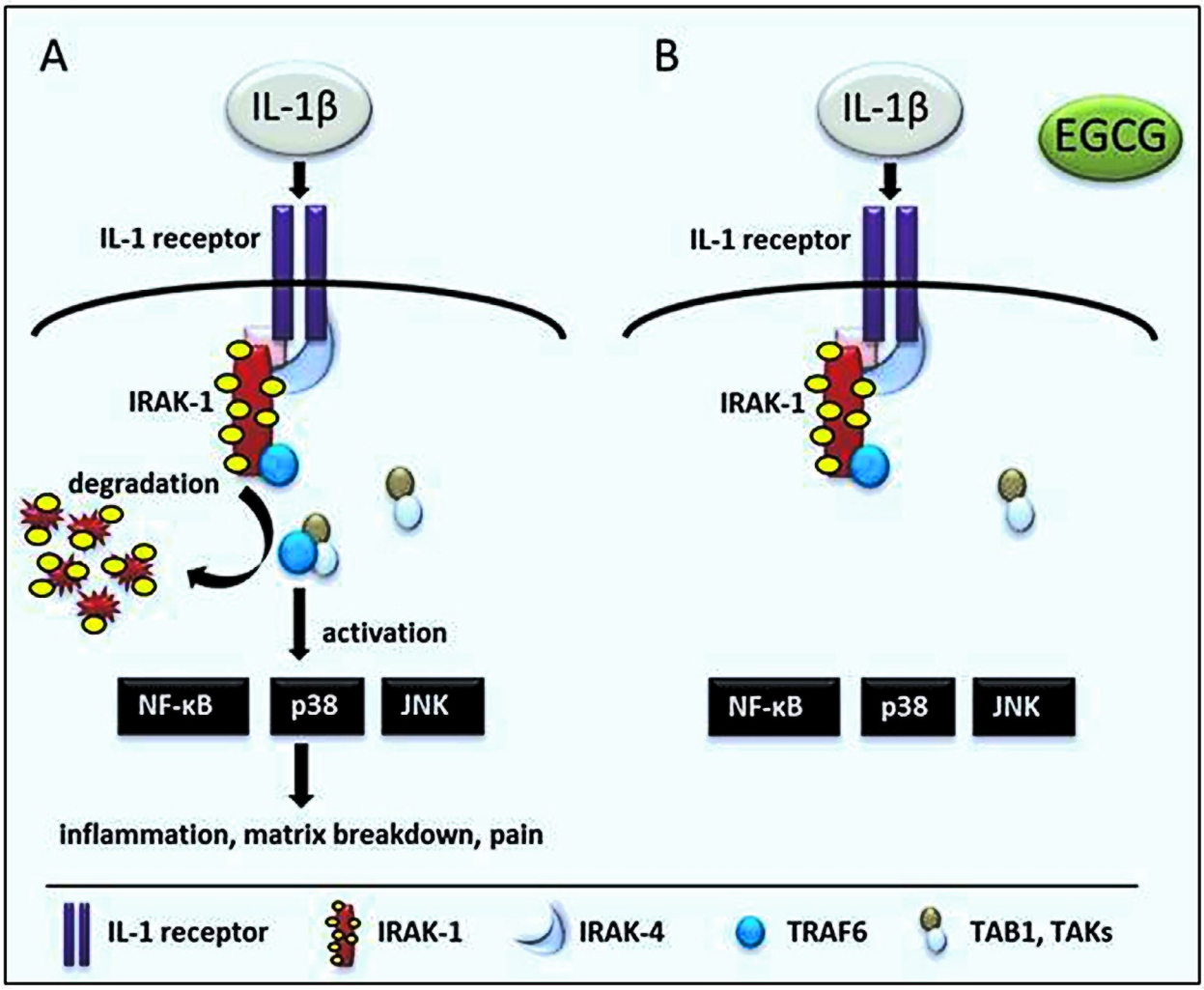

Fig. 5. Schematic of suggested mechanism of EGCG action in IVD cells. Upon IL-1β stimulation IRAK-1 is phosphorylated and degraded, which enables the formation of multiprotein complex (TRAF6/TAB1/TAKs) and activation of key stress-related effectors NF-κB, p38 and JNK which are further involved in the activation of inflammation, matrix breakdown and pain (**A**). EGCG blocks IRAK-1 degradation, formation of multiprotein complex and hence subsequent activation of NF-κB, p38 and JNK (**B**). TRAF6, TNF receptor-associated factor 6; TAK, TGF-beta activated kinase 1; TABs, TGF-beta activated kinase 1 binding proteins.

Table 4. The mean hind paw withdrawal thresholds for each group and time point.

	Baseline	day 2	day 7	day 14	day 21
sham	26±0	23.5±8.0	22.8±7.2	15.6±9.5	16.6±9.2
NP	26±0	10.2±6.3	9.5±6.3	5.7±4.7	9.1±6.2
NP + water	26±0	10.9±5.9	7±1.8	7.8±3.9	9.9±6.2
NP + EGCG 10 μM	26±0	15±7.3	11.5±7.5	15.4±8.5	17.8±8.9
NP + EGCG 100 μM	26±0	16.9±8.7	11.9±7.0	17.5±10.6	19.6±8.4

Table 5. Statistical analysis of von Frey filament test between the groups.

	sham	NP	NP + water	NP + EGCG 10	NP + EGCG 100
sham	x	$p = 0.0001$	$p < 0.0001$	non-significant	non-significant
NP	$p = 0.0001$	x	non-significant	$p = 0.0098$	$p = 0.0004$
NP + water	$p < 0.0001$	non-significant	x	$p = 0.0026$	$p < 0.0001$
NP + EGCG 10 μM	non-significant	$p = 0.0098$	$p = 0.0026$	x	non-significant
NP + EGCG 100 μM	non-significant	$p = 0.0004$	$p < 0.0001$	non-significant	x

EGCG inhibits pain behaviour *in vivo*

Animal behaviour in response to mechanical stimulation by von Frey filaments was compared between the NP + EGCG treatment groups (NP + EGCG 10 μM or NP + EGCG 100 μM), the NP group (NP + no treatment), the water group (NP + water) and the sham group (only facetectomy). Furthermore, the NP + EGCG treatment groups were compared between each other. In the NP groups without EGCG treatment, the mechanical withdrawal thresholds were significantly decreased for 28 d compared with the sham group, indicating that pain was evoked by application of NP tissue to the DRG. Over the experimental period, mechanical withdrawal thresholds upon EGCG treatment were significantly higher than in the NP group and NP + water group, and reached levels measured in the sham group. The mean hind paw withdrawal thresholds for each group at each time point are listed in Table 4. The exact *p*-values for each comparison are listed in Table 5. Results indicate that EGCG treatment reduced pain perception and this effect was independent of the applied concentration of EGCG (NP + EGCG 10 μM *vs*. NP + EGCG 100 μM = *n.s*.). Decrease in the mechanical withdrawal threshold observed in the sham group arises from facetectomy-related distress (Fig. 4).

Discussion

The herein presented study clearly demonstrates the anti-inflammatory and anti-catabolic properties of EGCG in human IVD cells *in vitro*, hence highlights its potential for the treatment of IVD-related back pain. The development of back pain involves both nociceptive and neuropathic pathophysiological mechanisms (Forster *et al.*, 2013; Morlion, 2011), with IVD degeneration being one of the major underlying causes. IVD degeneration is induced by phenotypic changes in the NP cells, leading to tissue degradation (Vo *et al.*, 2013), as well as to promotion of neoinnervation of the disc and hence the transmission of pain *in vivo* (Alimasi *et al.*, 2013; Richardson *et al.*, 2012). The evidence that IVD degeneration and low back pain are correlated with increased levels of pro-inflammatory cytokines is increasing (Wuertz *et al.*, 2012). Inflammatory processes are regulated, for example, by toll-like receptors (TLRs), which are primarily expressed on the immune cells as a first line of host defence, but have also been detected in the other cell types, including IVD cells (Ellman *et al.*, 2012; Yoon *et al.*, 2008). Recently, our group confirmed basal expression of TLRs in IVD cells and showed that expression of TLR2, 4 and 6 is increased during IVD degeneration (Klawitter *et al.*, 2014), thus further underlying the clinical relevance of inhibiting IVD inflammation. In this study, we demonstrated that 10 μM EGCG can significantly inhibit the mRNA expression of inflammatory and pain-related mediators IL-6, IL-8, TLR2, NGF, iNOS and COX-2 and matrix metalloproteinases MMP1, MMP3 and MMP13 in 2D and 3D IL-1β-stimulated IVD cell culture models. All of these markers have been described to be of relevance during IVD degeneration and inflammation and hence pain development (Gabr *et al.*, 2011; Wuertz and Haglund, 2013). Although all cells used in this study were isolated from degenerated discs, the difference in the expression of studied inflammation-related genes between the untreated control group and the EGCG-treated group is not significant, because basal expression of these genes decreases when cells are transferred to *in vitro* conditions (data not shown).

While this is the first IVD-related study, protective effects of EGCG and green tea extract have been reported in inflammatory arthritis (Shen *et al.*, 2012), where EGCG was shown to block IL-1β-induced expression of IL-6, IL-7, IL-8, IL-1β, TNF-α (Akhtar and Haqqi, 2011), MMP1 and MMP13 (Ahmed *et al.*, 2004), iNOS (Singh *et al.*, 2002) and COX-2 (Heinecke *et al.*, 2010), and these effects were linked mostly to NF-κB and JNK signalling pathways (Ahmed *et al.*, 2002; Akhtar and Haqqi, 2011; Rasheed *et al.*, 2009; Singh *et al.*, 2002; Singh *et al.*, 2003). In order to determine the underlying mechanisms in the IVD, potential pathways were identified from the literature (Ahmed *et al.*, 2002; Akhtar and Haqqi, 2011; Rasheed *et al.*, 2009; Singh *et al.*, 2002; Singh *et al.*, 2003) and the effects of EGCG on IRAK-1 degradation as well as NF-kB and MAPK activity in IVD cells were analysed. We propose that EGCG acts as an IL-1 receptor antagonist in IL-1β-stimulated IVD cells. Our suggested mechanism of action, which is illustrated in Fig. 5, is through the inhibition of IRAK-1 degradation. As IL-1 receptors do not possess intrinsic kinase activity, they rely on the recruitment and activation of intrinsic kinases (IRAKs) (Janssens and Beyaert, 2003). Upon phosphorylation, IRAK-1 is ubiquitinated and degraded, which initiates the formation of a cytosolic multi-protein complex. The IRAK-1-activated protein complex is involved in the phosphorylation of the inhibitor of κB kinase (IKK) as well as of MAPK kinases 3/4/6, leading to the activation of NF-κB and JNK/p38 MAPKs, respectively (Burns *et al.*, 2003; Janssens and Beyaert, 2003). In this study, we demonstrate inhibition of IRAK-1 degradation in IVD cells, similar to IL-1β-stimulated OA chondrocytes (Akhtar and Haqqi, 2011). As a result, activity of NF-κB was decreased in IL-1β-stimulated EGCG-treated IVD cells. This effect was observed after 1 h, since NF-κB is a protein complex that functions as a "rapid-acting" transcription factor and as such regulates the transcription of more than 150 genes related to stress responses, including MMPs (Elliott *et al.*, 2002; Liacini *et al.*, 2003; Mengshol *et al.*, 2000; Wuertz *et al.*, 2012).

The inhibition of IRAK-1 degradation is accompanied by a decrease in the activity of its downstream effectors p38 and JNK. p38 kinase is a MAPK family member that is activated in various stress conditions, such as UV irradiation, hypoxia, oxidative stress and inflammation, and regulates the expression of many stress-related genes, leading either to restoration of homeostasis or to alteration of cellular functions and apoptosis. Recently it was published that blockage of p38 MAPK activity in rabbit (Studer *et al.*, 2008) and human (Studer *et al.*, 2007) NP cells reduces IL-1β-induced NO and prostaglandin E-2 accumulation (Kim *et al.*, 2012; Studer *et al.*, 2008) and partially restores proteoglycan synthesis (Studer *et al.*, 2008). Using the small-molecule inhibitor SB203580, we

confirmed that not only iNOS and COX-2 but also MMP1 and MMP13 are p38 MAPK target genes in human IVD cells (similar to bovine IVD cells (Seguin *et al.*, 2006)), hence indicating that the p38 pathway may play an essential role in ECM degradation and nociception in the disc. Importantly, the concomitant regulation of MMP1 and MMP13 suggests that EGCG may slow down MMP-induced collagen loss in all stages of IVD degeneration, as MMP1 has been shown to be significantly increased in severely degenerated NPs whereas MMP13 is increased in earlier stages of NP degeneration (Le Maitre *et al.*, 2004; Vo *et al.*, 2013). The expression of other studied genes (IL-6, IL-8, MMP3, TLR2 and NGF) is regulated differently in IVD cells, although p38 may co-operate too. In the context of nociception, the observed decrease in the prostaglandin H synthase COX-2 can be specifically relevant as it catalyses the initial step in the conversion of arachidonic acid to prostaglandins, which have a variety of physiological effects including sensitisation of spinal neurons to pain (Studer *et al.*, 2008). Similarly, NOS plays an important role in the processing of pain (Meller and Gebhart, 1993), so that the reduction of iNOS expression by EGCG should be highlighted. Interestingly, it was recently reported that intrathecal administration of EGCG could produce an antiallodynic effect against spinal nerve ligation-induced neuropathic pain, mediated by blockade of neuronal NOS protein expression and inhibition of nitric oxide (NO) (Choi *et al.*, 2012). As described above, our *in vitro* results suggest that EGCG-driven p38-mediated inhibition of COX-2 and iNOS expression may be helpful for nociceptive as well as neuropathic pain reduction. In order to monitor changes in pain behaviour upon EGCG treatment *in vivo,* we used an animal model in which NP-mediated pain is simulated by the application of NP tissue to the DRG, thus representing typical radiculopathic pain (Kato *et al.*, 2008; Sasaki *et al.*, 2007; Tachihara *et al.*, 2008). EGCG treatment was able to prevent the threshold reduction and thus the pain-related behaviour. The full recovery to the sham threshold level was observed on day 14 in both EGCG treatment groups. Our results correspond to recent findings using the rat model of neuropathic pain (sciatic nerve constriction) where intrathecal injection of EGCG markedly improved pain behaviour in rats and decreased the expression of TLR4, NF-κB, high-mobility group protein B1 (HMGB1), tumour necrosis factor alpha (TNF-α) and IL-1β in the spinal cord (Kuang *et al.*, 2012). In humans, back pain is however caused by complex interactions of biological, psychological and social factors and can be considered as a syndrome with both nociceptive and neuropathic components (mixed pain) (Forster *et al.*, 2013). Nociceptive nerves are a major transmitter of discogenic back pain (Freemont, 2009), therefore alteration of nerve ingrowth into the disc by EGCG-mediated inhibition of neurotrophins can modulate the development of acute and chronic nociceptive pain. On the other hand, discogenic radiculopathy occurs when functional, vascular and morphological changes of the nerve root are activated, *e.g.* by disc-related inflammation, which leads to intraradicular fibrosis and nerve fibre atrophy (Freemont, 2009). From this point of view and according to our *in vivo* results, EGCG-mediated inhibition of the inflammatory response of NP tissue may be beneficial also against the development of disc-related radiculopathy. Based on these results, we hypothesise that EGCG may reduce pain behaviour *in vivo* by inhibiting the expression of cytokines and pain-related inflammatory mediators, similar to the mechanism observed in our *in vitro* cell culture study.

This study however has certain limitations; firstly, the mechanism of EGCG action is not entirely clear. During disc degeneration, the regulation and function of the IL-1-mediated pathway is altered: IL-1α and IL-1β isoforms as well as IL-1 receptor are synthesised in larger quantities compared to healthy tissue, whereas the expression of IL-1 receptor antagonist is unchanged (Le Maitre *et al.*, 2007b). This imbalance activates the IL-1 pathway in NP tissue, which leads to the induction of the expression of MMPs, ADAMTs, as well as to enhanced angiogenesis and neurogenesis (Freemont, 2009). Although our study does not address the exact mechanism of how EGCG influences the IL-mediated pathway, it provides evidence that EGCG can act as an IL-1 pathway antagonist, therefore can possibly help to restore tissue homeostasis *in vivo*. The second limitation is that NP cells were isolated from donors with different medical conditions due to the limited number of available biopsies. In addition, exact separation of NP and AF tissue (which was performed in the surgical as well as the laboratory setting) can be challenging in degenerated material so that certain impurities are possible. Nevertheless, all donors respond similarly. Finally, the effect of EGCG at the tissue level (matrix degradation) was not analysed in this study.

Considering the anti-inflammatory, anti-catabolic and analgesic properties of EGCG presented in this study, we suggest that EGCG could in the future supplement or even substitute drugs which are used currently for the treatment of low back pain and disc degeneration. However, the best mode of application is still a matter of debate. While oral application has been considered for diseases such as obesity or diabetes, large *in vitro*/*in vivo* discrepancy was observed (Mereles and Hunstein, 2011) due to the low bioavailability of EGCG, with an elimination half-life in plasma of around 3.5 h (Lee *et al.*, 2002). For the treatment of back pain, oral application of EGCG is certainly not a promising approach because the inflammatory processes within the IVD need to be targeted directly. Therefore, local application (*e.g.* intradiscal or epidural injection) will be required, which could be done in conjunction with other surgical or regenerative interventions or independently, *i.e.* as an early, non-invasive treatment. However, it is likely that the effects of non-modified (free) EGCG will be less pronounced *in vivo* compared to *in vitro* because of its low stability, which can be further influenced by extrinsic conditions such as type of storage and intrinsic factors such as Ca^{2+} and Mg^{2+} ions or antioxidant concentration (Mereles and Hunstein, 2011). Encapsulation of EGCG in polymeric carriers represents a promising strategy to increase its bioavailability and release period. In fact, different types of polymeric carriers administered orally or intravenously

were demonstrated to significantly increase the stability and efficiency of EGCG in cancer therapy (Wang *et al.*, 2012; Wang *et al.*, 2013), showing their potential to be used also inside the IVD. However, the disc-specific environment (high level of proteoglycan, lower pH in degenerated disc, low number of cells) should be taken into account when choosing a carrier and delivery method for EGCG. The efficacy of these respective EGCG slow-release systems will have to be determined *in vitro* using cell or organ culture studies (by detecting inflammation) as well as in appropriate *in vivo* models (by detecting pain sensation). Despite the remaining challenges, EGCG offers several potential clinical advantages: it is globally available, it is inexpensive to isolate, it was reported to be safe and well tolerated, and clinically active concentrations can be reached by its oral administration or by its modification (Bode and Dong, 2009; Singh *et al.*, 2011).

Conclusion

In this study, we examined the effects of the bioactive polyphenol from green tea, epigallocatechin 3-gallate, on IVD cells cultured in 2D and 3D *in vitro* and on IVD-related radiculopathic pain *in vivo*. We have described its anti-inflammatory and anti-catabolic effects and the benefit of EGCG in the reduction of both nociceptive and neuropathic disc-related pain. We identified IRAK-1 and NF-κB/p38/JNK signalling pathways to be modulated by EGCG and we provide evidence that this contributes to the observed effects. Although precise mechanisms of EGCG action and its direct targets in IVD cells still need to be elucidated, we showed promising therapeutic potential of EGCG in the treatment of disc-related inflammation and back pain in degenerative disc disease.

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OK performed cell culture experiments, statistical analysis and helped to draft the manuscript. MS performed animal experiments, statistical analysis and helped to draft the manuscript. JK participated in study design, provided clinical samples and medical scientific input and helped to draft the manuscript. OH participated in study design, provided clinical samples and medical scientific input and helped to draft the manuscript. SK helped with study design and coordination and helped to draft the manuscript. SJF conceived funding of the study, helped with study design and coordination and helped to draft the manuscript. KW conceived funding, designed and coordinated the study and helped to draft the manuscript. All authors approved a final version of manuscript. Funding for this research project was provided by the European Union through a Marie Curie action (FPT7-PITN-GA-2009-238690-SPINEFX) and Theodor und Ida Herzog-Egli Foundation. The authors would like to thank Ms. Greutert for technical assistance. We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Discussion with Reviewers

Reviewer I: If the effects of the EGCG are short lived and it has a half-life in plasma of only 3.5 h, how can we explain that effect *in vivo* was not seen until week 2?

Authors: The half-life of EGCG has been reported to be 3.5 h in human plasma. In rodent animal models, the activity of intracellular and extracellular enzymes can be different, *e.g.* due to the efficiency of peroxidases that decrease the activity of EGCG by oxidation (Patry *et al*., 2003). In our animal experiment, the improvement in the hind paw withdrawal threshold is not observed until the day 7 after the application of EGCG. One explanation for the delayed effect in rats can be a difference in the occurrence and activity of EGCG-degrading enzymes between rat and human tissue. The effect of EGCG can decrease *via* its oxidation by myeloperoxidase and eosinophil peroxidase, which are secreted by activated leukocytes involved in the clearance of displaced NP tissue. A second reason for delayed anti-inflammatory response in this animal model can be the anti-catabolic activity of EGCG itself, which can initially inhibit the MMPs from leukocytes that are involved in the clearance of displaced NP tissue. Furthermore, EGCG can mediate receptor/enzyme activity by inhibiting inflammation-related signalling pathways, which, according to our *in vitro* results, activate the cellular response at the level of gene expression within 24 h. Therefore, the observed delayed analgesic effect *in vivo* can occur partially due to a general inhibition of inflammation in the displaced NP tissue, rather than due to the quick blockage of pain development (nerve root reaction).

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